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Dynamics of biochemical components, lipid classes and energy values on gonadal development of *R. philippinarum* associated with the temperature and ingestion rate

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Abstract

This study evaluates the effect of temperature, coupled with ingestion rate, on the dynamics of biochemical components and lipid classes in *R. philippinarum*. The data are discussed with regard to sexual development and energy balance. Experimental protocol developed in the present study used two groups of the clam *R. philippinarum*: L (temperatures of 14 °C and 18 °C) and H (temperatures of 18 °C and 22 °C). The intra-group ingestion level was similar, although the ingestion level of the clams in the group H was 2.4 times higher than group L. We observed that *R. philippinarum* conditioned at 18 °C (18L) shows higher protein content, furthermore an important loss of organic weight was observed after 48 days. In such a situation, the clams use their own reserves (carbohydrates and glycogen) for sexual development while in situations without food stress (positive energy balance) and low temperature (14 °C) an accumulation of reserves is produced. Strikingly dissimilar behaviour in biochemical composition was observed for the 18H and 22H treatments, both with a positive energy balance. Despite similar protein content, the highest levels of carbohydrates were observed at the lower temperature (18 °C). Glycogen was also higher for the 18 °C treatment, although the differences were significant only in the males. Although the total lipids in *R. philippinarum* showed no significant differences in any treatment, they became apparent and related to sex when considering the individual lipid classes. There was no variation in lipid classes in the males between the 14L and 22H treatments despite the large disparity in the degree of sexual development. However, in the females significant differences in lipid classes (phospholipids, triglycerides) were observed. The results of this study show that a positive energy balance permits *R. philippinarum* gonadal development and accumulation of reserves both in low and high temperature conditions. In low temperature situations, gonadal development is slower and the energy reserves are accumulated in the form of carbohydrates. When the clams are conditioned at high temperatures, gonadal development is fast and complete, carbohydrates are consumed and lipids are accumulated.

Keywords: Clams; Gonadal development; Temperature; Ingestion; Biochemical composition; Lipid classes

1. Introduction

Many studies have related changes in the biochemical composition of bivalves with the reproductive cycle, mostly in the natural environment. Widdows and Bayne (1971) observed high contents of glycogen in *Mytilus edulis* during summer when energetic demands are low. Reserves of lipids and proteins were also elevated. During autumn and winter, the energetic demand increased and glycogen fell to minimum levels. In other species of mollusc (i.e. *Mytilus galloprovincioalis*, *Ruditapes philippinarum*, and *Ruditapes decussatus*) a tight relationship between biochemical composition and reproductive cycle has also been observed (Beninger and Lucas, 1984; Bressan and Marin, 1985).

In general, changes in biochemical components are closely linked to the state of sexual maturity of the mollusc and to energy supply, either directly from ingested food or from previously stored reserves (Sastry, 1979; Navarro et al., 1989). Carbohydrates, particularly glycogen, are considered to be the main energy source in adult marine bivalves, and are important

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Table 1
Organic weight, biochemical composition and energy content of *R. philippinarum* at the start of the experimental period

	% OW	mg ind^{-1}	kJ ind^{-1}
Organic weight		345.5±56.9	
Protein	72.9±3.0	251.8±10.3	4.5±1.0
Carbohydrates	16.2±3.2	56.1±11.1	1.0±0.2
Glycogen	5.7±1.5	19.7±3.1	0.3±0.1
Lipids	10.8±0.9	37.4±2.8	1.3±0.3

%OW: relative percentage to the total organic matter.
Values are means±S.D.

for gamete formation and maintenance of adult condition during periods of nutritive stress or in winter (De Zwaan and Zandee, 1984; Gabbott, 1975). Variations in carbohydrate content show an inverse relationship with the state of gonad maturity (Beninger, 1982). According to Beninger and Lucas (1984), lipids form part of the reserves during periods of nutritional deficiency and are an important component of bivalve oocytes (Holland, 1978). Their maximum levels thus occur in the pre-spawning period (Taylor and Venn, 1979). Finally, proteins quantitatively constitute the largest fraction in the oocytes and other soft tissues, and assume the role of energy providers during sexual maturation (Holland, 1978).

Temperature and food availability are the environmental factors which most affect the reproductive cycle of bivalves and, consequently, the mobilisation of biochemical reserves (Sastry, 1979). The effects of both factors have been observed to become superimposed in the natural environment (Holland and Chew, 1974; Beninger and Lucas, 1984; Laruelle et al., 1994; Xie and Burnell, 1994).

Few studies have been carried out under controlled conditions which follow the effects of temperature and food availability or which adequately differentiate their impact (Mann, 1979; Heasman et al., 1996; Martínez and Pérez, 2003). Other works have not traced biochemical compositional changes in detail (Borcherding, 1995; Martínez et al., 2000). Additionally, the majority of studies investigating the temperature influence on bivalve reproduction offered the same food quantity, with no consideration of the temperature effect on physiological rates and energetic balance (Saucedo et al., 2001; Martínez and Pérez, 2003).

Experimental protocol developed in the present study uses 2 groups of the clam *R. philippinarum*: L (temperatures of 14 °C and 18 °C) and H (temperatures of 18 °C and 22 °C). The intra-group ingestion level is similar, although the inter-group ingestion level is different. Previous results indicate that with similar quantities of ingested food, temperature differences of 14 °C vs 18 °C and 18 °C vs 22 °C do not impact significantly on the speed of gonadal development but do affect the energetic balance (SFG) (Delgado and Pérez-Camacho, 2007). The present study investigates the effect of temperature, coupled with ingestion rate, on the dynamics of biochemical components and lipid classes in *R. philippinarum*. The data are discussed in the context of sexual development, according to Delgado and Pérez-Camacho (2007).

2. Materials and methods

2.1. Biological material

The clams were obtained from a beach in the Ría de Arosa (NW Spain), with an average initial length (*L*) of 37.15± 0.26 mm. Tables 1 and 2 detail their biochemical composition and lipid classes at the start of the experiment. At the start of the experimental period clams were in their resting phase or at initial phase of gonadal development with a gonadal occupation index (GOI) below 10% in females, and between 7 and 12% in males. No statistically significant differences in initial GOI values appeared between the various experimental groups ($p>0.05$).

2.2. Design and experimental conditions

The experiments were performed using adult specimens of *R. philippinarum* in 12 L plastic tanks. Natural filtered (1 μm) sea-water (33‰ salinity) was used in an initial flow-through circuit with a water flow dependent on the food ingestion rate.

The clams were fed with the microalgae *Isochrysis galbana* clone T-ISO. The microalgae were firstly cultured in 6 L jars followed by 1000 L tanks. Walne medium (Walne, 1966) and industrial fertiliser were used for the jar and tank cultivations, respectively. The microalgae were harvested during the stationary growth phase. The food was supplied to the circulating water with a variable-flow peristaltic pump (Pérez-Camacho et al., 2003).

Since one of the experimental requirements was to maintain identical ingestion rates (IR) at different temperatures, and taking into account the influence of temperature on the IR of the clams, given the noticeable difference in IR between clams kept at 14 °C and those kept at 22 °C, if the clams kept at 22 °C has the same IR as those kept at 14 °C then this would lead to an extremely negative energy balance in the former, which would have a considerable effect on gonadal development (Delgado and Pérez-Camacho, 2005).

In order to minimise the effect of the differences between energy balances at the different temperatures, the comparison of temperatures was divided into two sections, 14 °C–18 °C and 18 °C–22 °C. Thus, two levels of IR were assayed, referred to as low (L) and high (H), whilst three different temperatures were used, 14 °C, 18 °C and 22 °C, giving a total of 4 experimental conditions: 14L, 18L, 18H and 22H (with 120 specimens per experimental condition).

The daily food ration was 750 μg organic weight of phytoplankton per g clam live weight for experimental condition 14L,

Table 2
Lipid class content of *R. philippinarum* at the start of the experimental period

	% DW	mg ind^{-1}
Phospholipids	3.1±0.7	12.1±2.5
Sterol ester+waxes	0.2±0.0	0.7±0.2
Triacylglycerols	0.0±0.0	0.0±0.0
Free fatty acids	0.2±0.1	0.7±0.2
Sterols	0.6±0.1	2.3±0.4

%DW: relative percentage to the total dry matter.
Values are means±S.D.

and 1500 μg for experimental condition 18H, these being rations which did not restrict daily ingestion rates (IR). IRs were measured daily and the rations for other two experimental conditions (18L and 22H) were modified by adjusting the input of food to the experimental vessels so as to make the IR for conditions 18L and 22H coincide with those of 14L and 18H, respectively (Delgado and Pérez-Camacho, 2007).

As a result of this experimental design it was possible to harmonize ingestion rates between the clams in each group of experiments, with ingestion rates of between 1000–1100 μg organic weight (OW) of phytoplankton per g clam live weight (LW) day^{-1} for the 18H–22H group, and between 470–550 μg OW day^{-1}.g LW^{-1} for the 14L–18L group.

The total conditioning period was 70 days, and sampling was performed at the start of the experiment, and on day 28, 48 and 70. Samples for biochemical analysis were taken on day 48. At this point the clams exhibited gonadal development without any observable spawning episodes.

2.3. Analytical methods

Biochemical analyses were performed individually, and therefore the values shown in the present manuscript correspond to the mean of 6 independent analyses by sex. Proteins were calculated using the method described by Lowry et al. (1951) after alkaline hydrolysis with NaOH (0.5 N/30 °C). Carbohydrates were quantified as glucose by the phenol–sulphur method (Strickland and Parsons, 1968). Glycogen was also quantified as glucose after precipitation with 100% ethanol.

Lipids were first extracted with chloroform:methanol (1:2). After centrifugation (3246 g), the precipitate was extracted with chloroform:methanol (2:1). Both supernatants were washed with chloroform:methanol:water (8:4:3) as described by Fernández-Reiriz et al. (1989). The solvents contained 0.05% butylated hydroxytoluene (BHT). Storage until further processing was carried out under nitrogen at −70 °C. Total lipids were determined gravimetrically by evaporation of the solvent in the purified extract on aluminium sheets at 60–80 °C.

The lipid classes were determined by thin layer chromatography (TLC) using 20×20 mm silica-gel plates (Merck 16486) with a thickness of 0.25 mm. The chromatographic plates were developed following the technical method of Freeman and West (1966). The samples were placed on the plates with an automated instrument designed for TLC (Camag 27220), and developed with a 10% $CuSO_4$ and 0.85% H_3PO_4 solution heated to 180 °C (Bitman and Wood, 1982). The developed plates were read with a Scanner-Densitometry (Shimadzu CS9000), equipped with a monochromatic 370 nm bulb (0.4×0.4 mm). The scanner read the TLC trace in zig-zag formation or "complete migration", from a baseline automatically graded to zero (0). Standards employed for the quantitative analysis of the sterol and wax esters, sterols, free fatty acids and triglycerides were cholesterol palmitate, cholesterol, palmitic acid and tripalmitin (Sigma), respectively. The phospholipid standard was obtained from the clam *R. philippinarum*.

The results of the biochemical analyses, expressed in units of weight per individual, were transformed into their energy equivalents using the following factors: 18, 17.2 and 35.2 kJ g^{-1} of proteins, carbohydrates and lipids, respectively (Beukema and De Bruin, 1979).

2.4. Statistical analysis

Percent composition data were transformed by angular transformation (arcsin √percentage) prior to analyses to ensure normality. In cases where data transformations were insufficient to produce the homogeneity of the variances, a rank analysis of the variance was carried out with the Kruskal–Wallis test (Snedecor and Cochran, 1980; Zar, 1984). A two-way ANOVA was used to determine the effect of sex and temperature on biochemical composition and lipid classes (Snedecor and Cochran, 1980; Zar, 1984). One-way analysis of variance (ANOVA) was followed by a Tukey test with a significance level of $P<0.05$ in order to examine possible differences due to temperature on the biochemical composition and lipid classes within the groups of homogenous ingestion (14 °CL vs 18 °CL and 18 °CH vs 22 °CH) and sex.

3. Results

3.1. Organic weight and biochemical composition — effect of sex and temperature on organic weight and biochemical composition

3.1.1. 14L vs 18L

The two-way ANOVA (Table 3) showed a significant effect of temperature and sex–temperature interaction on organic

Table 3
Results of the 2 way ANOVA testing the effect of sex and temperature on the organic weight and the biochemical composition in *Ruditapes philippinarum* after 48 experimental days (group: 14 °C L vs 18 °C L)

Source	Sum of square	*df*	Mean square	*F* ratio
Organic weight				
Sex	3242.96	1	3242.96	1.03ns
Temperature	137929.98	1	137929.98	43.80***
Sex × temperature	12650.06	1	12650.06	4.02*
Error	40934.05	13	3148.77	
Protein				
Sex	10.32	1	10.32	3.58ns
Temperature	104.83	1	104.83	36.32***
Sex × temperature	1.00	1	1.00	0.35ns
Error	37.53	13	2.89	
Carbohydrates				
Sex	6.92	1	6.92	0.23ns
Temperature	105.24	1	105.24	24.08***
Sex × temperature	1.21	1	1.21	0.28ns
Error	56.82	13	4.37	
Glycogen				
Sex	1.96	1	1.96	0.71ns
Temperature	52.76	1	52.76	19.17**
Sex × temperature	0.16	1	0.16	0.06ns
Error	35.77	13	2.75	
Lipids				
Sex	2.60	1	2.60	2.57ns
Temperature	8.10	1	8.10	7.99*
Sex × temperature	0.06	1	0.06	0.05ns
Error	13.16	13	1.01	

ns, not significant; *$p<0.05$; **$p<0.01$; ***$p<0.001$.

Table 4
Organic weight (mg ind^{-1}), biochemical composition (%OW) of *R. philippinarum* after 48 experimental days

	Females		Males		Females		Males	
	14 °C L	18 °C L	14 °C L	18 °C L	18 °C H	22 °C H	18 °C H	22 °C H
Organic weight (mg ind^{-1})	467.6±80.9	221.7±81.3	381.5±59.6	249.9±9.0	460.9±94.7	449.0±73.2	472.4±67.7	364.5±68.8
Protein (%OW)	68.8±2.5^{a}	77.6±3.2^{b}	72.2±3.3^{a}	79.2±1.4^{b}	71.1±3.5^{a}	73.4±2.4^{a}	73.2±1.4^{a}	75.9±1.5^{b}
Carbohydrates(%OW)	17.7±1.6^{a}	10.7±1.8^{b}	15.4±4.3^{a}	9.9±0.4^{b}	12.5±2.6^{a}	9.8±0.3^{b}	13.7±2.3^{a}	10.1±1.2^{b}
Glycogen(%OW)	5.5±1.5^{a}	2.7±0.5^{b}	4.8±1.9^{a}	2.5±0.3^{b}	3.5±0.7^{a}	3.0±0.2^{a}	3.8±0.9^{a}	2.7±0.3^{b}
Lipids(%OW)	13.5±0.9^{a}	11.7±1.5^{a}	12.4±1.1^{a}	10.9±1.0^{b}	16.4±1.9^{a}	16.7±2.2^{a}	13.1±0.9^{a}	14.0±1.3^{a}

%OW: relative percentage to the total organic matter. Values are given as means±S.D.
Mean values within the same group and same sex (see explain in the text) with different superscript letters are significant different ($p<0.05$).

weight accounted for more than 70.8% of the variance for the factor temperature. Temperature was the only significant factor affecting the content of proteins, carbohydrates, glycogen and total lipids, accounting for 68.2%, 61.8%, 52.2% and 33.9% of the variance, respectively.

The one-way ANOVA comparing groups (14 °CL vs 18 °CL) of the same sex and the Tukey test established that the protein content in males and females was greater ($p<0.05$) in clams cultured at 18 °C than those at 14 °C. Conversely, the carbohydrate ($p<0.01$) and glycogen ($p<0.05$) contents were higher in the clams cultured at 14 °C. Differences in total lipids were associated with the sex. Females showed no significant differences ($p>0.05$) between temperatures, although the lipid content of the male clams cultured at 14 °C were higher ($p<0.05$) than those at 18 °C (see Table 4).

3.1.2. 18H vs 22H

The two-way ANOVA (Table 5) showed that temperature and sex had no significant effect on organic weight, but did affect protein content (16.2 and 20.6%, respectively). Temperature was the only significant factor affecting the carbohydrate and glycogen content (46.3 and 36.1%, respectively) whereas sex was the factor explaining the greatest percentage of the variance in total lipids (88.4%).

The one-way ANOVA comparing groups (18 °C°H vs 22 °CH) of the same sex and the Tukey test established that the females showed significant differences only in carbohydrate ($p<0.05$), with the higher percentages at 18 °C as compared to 22 °C. With the exception of total lipids ($p>0.05$), significant differences in the males were observed in all components studied. The protein content was greater ($p<0.05$) at 22 °C,

Table 5
Results of the 2 way ANOVA testing the effect of sex and temperature on the organic weight and the biochemical composition in *Ruditapes philippinarum* after 48 experimental days (group: 18 °C H vs 22 °C H)

Source	Sum of square	*df*	Mean square	*F* ratio
Organic weight				
Sex	6521.55	1	6521.55	1.05ns
Temperature	17547.24	1	17547.24	2.83ns
Sex × temperature	11277.61	1	11277.61	1.82ns
Error	99034.50	16	6189.66	
Protein				
Sex	10.36	1	10.36	4.11*
Temperature	13.18	1	13.18	5.22*
Sex × temperature	0.16	1	0.16	0.06ns
Error	40.39	16	2.52	
Carbohydrates				
Sex	2.08	1	2.08	0.81ns
Temperature	37.96	1	37.96	14.81**
Sex × temperature	0.99	1	0.99	0.39ns
Error	41.00	16	2.56	
Glycogen				
Sex	0.07	1	0.07	0.09ns
Temperature	7.71	1	7.71	10.06*
Sex × temperature	1.33	1	1.33	1.73ns
Error	12.27	16	0.77	
Lipids				
Sex	28.11	1	28.11	15.80**
Temperature	1.27	1	1.27	0.71ns
Sex × temperature	0.31	1	0.31	0.17ns
Error	28.46	16	1.78	

ns, not significant; *$p<0.05$; **$p<0.01$; ***$p<0.001$.

Table 6
Results of the 2 way ANOVA testing the effect of sex and temperature on the lipid classes in *Ruditapes philippinarum* after 48 experimental days (group: 14 °C L vs 18 °C L)

Source	Sum of square	*df*	Mean square	*F* ratio
Phospholipids				
Sex	26.06	1	26.06	32.24***
Temperature	11.38	1	11.38	14.08**
Sex × temperature	8.33	1	8.33	10.31*
Error	10.51	13	0.81	
Sterol ester + waxes				
Sex	7.91	1	7.91	31.97***
Temperature	1.21	1	1.21	4.89*
Sex × temperature	1.94	1	1.94	7.85**
Error	3.21	13	0.25	
Triacylglycerol				
Sex	28.94	1	28.94	75.68***
Temperature	9.05	1	9.05	23.67**
Sex × temperature	0.01	1	0.01	0.04ns
Error	3.06	13	0.38	
Free fatty acid				
Sex	0.15	1	0.15	0.36ns
Temperature	0.18	1	0.18	0.43ns
Sex × temperature	0.07	1	0.07	0.16ns
Error	5.62	13	0.43	
Sterols				
Sex	0.87	1	0.87	5.27*
Temperature	0.13	1	0.13	0.81ns
Sex × temperature	0.83	1	0.83	5.08*
Error	2.13	13	0.16	

ns, not significant; *$p<0.05$; **$p<0.01$; ***$p<0.001$.

Table 7
Lipid class content of *R. philippinarum* after 48 experimental days

	Females (%DW)		Males (%DW)		Females (%DW)		Males (%DW)	
	14 °C L	18 °C L	14 °C L	18 °C L	18 °C H	22 °C H	18 °C H	22 °C H
Phospholipids	6.3±1.2^{a}	3.8±0.5^{b}	3.3±0.3^{a}	3.1±0.7^{a}	4.4±0.3^{a}	3.4±0.7^{b}	3.9±0.9^{a}	3.6±0.3^{a}
Sterol ester+waxes	0.5±0.2^{a}	0.2±0.1^{b}	0.1±0.0^{a}	0.1±0.0^{a}	0.3±0.1^{a}	0.4±0.1^{a}	0.2±0.0^{a}	0.1±0.0^{b}
Triacylglycerols	0.8±0.1^{a}	0.4±0.2^{b}	0.1±0.0^{a}	0.0±0.0^{b}	1.8±0.8^{a}	2.0±0.7^{a}	0.2±0.1^{a}	0.0±0.0^{b}
Free fatty acids	0.2±0.1^{a}	0.2±0.1^{a}	0.2±0.1^{a}	0.2±0.1^{a}	0.2±0.0^{a}	0.4±0.1^{b}	0.2±0.1^{a}	0.3±0.1^{a}
Sterols	0.9±0.1^{a}	0.7±0.1^{b}	0.6±0.1^{a}	0.7±0.1^{a}	0.5±0.1^{a}	0.7±0.1^{a}	0.6±0.1^{a}	0.7±0.1^{a}

%DW: relative percentage to the total dry matter. Values are given as means±S.D.
Mean values within the same group and same sex (see explain in the text) with different superscript letters are significant different ($p<0.05$).

whereas the carbohydrate and glycogen content were greater ($p<0.05$) in the 18 °C clams (see Table 4).

3.2. Lipid classes

3.2.1. 14L vs 18L

The two-way ANOVA (Table 6) showed a significant effect of sex, temperature and the sex–temperature interaction on phospholipids and esters of sterols+waxes. Sex explained the highest percentage of variance in the content of the latter compounds (46.3% and 55.4%, respectively). A significant effect of sex and temperature was observed in the triglycerides, with the former explaining the greater percentage of variance (70.5%). The ANOVA showed a significant effect of sex and the sex–temperature interaction on sterols, both factors explaining a similar variance (~21%). No factor was significant for free fatty acids ($p>0.05$).

Table 8
Results of the 2 way ANOVA testing the effect of sex and temperature on the lipid classes in *Ruditapes philippinarum* after 48 experimental days

Source	Sum of square	*df*	Mean square	*F* ratio
Phospholipids				
Sex	0.27	1	0.27	0.35ns
Temperature	4.45	1	4.45	5.82*
Sex×temperature	1.11	1	1.11	1.46ns
Error	10.69	13	0.76	
Sterol ester+waxes				
Sex	2.80	1	2.80	9.99**
Temperature	0.00	1	0.00	0.01ns
Sex×temperature	0.65	1	0.65	2.30ns
Error	3.65	13	0.28	
Triacylglycerol				
Sex	135.86	1	135.86	84.11***
Temperature	1.90	1	1.90	1.18ns
Sex×temperature	5.69	1	5.69	3.52ns
Error	16.15	13	1.61	
Free fatty acid				
Sex	0.02	1	0.02	0.07ns
Temperature	1.94	1	1.94	5.67*
Sex×temperature	0.41	1	0.41	1.19ns
Error	3.76	13	0.34	
Sterols				
Sex	0.22	1	0.22	1.03ns
Temperature	0.97	1	0.97	4.57*
Sex×temperature	0.00	1	0.00	0.01ns
Error	2.98	13	0.21	

ns, not significant; *$p<0.05$; **$p<0.01$; ***$p<0.001$.
(group 18 °CH vs 22 °CH).

The one-way ANOVA comparing groups (14 °CL vs 18 °CL) of the same sex and the Tukey test establish that *R. philippinarum* females treated at 14 °C showed a significantly higher content ($p<0.05$) of phospholipids, triglycerides, sterol ester+waxes and sterols. For the males, only the triglycerides presented significant differences, with the highest content in the 14 °C clams ($p<0.05$) (Table 7).

3.2.2. 18H vs 22H

The two-way ANOVA showed a significant effect of temperature on the phospholipids, free fatty acids and sterols content and explained 26.9, 31.6 and 23.3% of the variance, respectively. Sex was the only significant factor for esters of sterols+waxes and triglycerides, explaining 39.5 and 85.1%, respectively (Table 8).

The one-way ANOVA comparing groups (18 °CH vs 22 °CH) of the same sex and the Tukey test establish that *R. philippinarum* females treated at 18 °C presented higher contents ($p<0.05$) of phospholipids than those at 22 °C, whereas the free fatty acids were higher at 22 °C ($p<0.05$). With regard to the males, significantly higher values of esters of sterols+waxes and triglycerides were observed in the 18H clams (Table 7).

4. Discussion

Food availability and temperature are the main factors affecting growth and reproduction in bivalves. The effect of these variables is complex and depends specifically on acquisition and energy expenditure in the natural environment (Bayne and Newell, 1983). The accumulation of reserves, the allocation of stored energy to somatic growth or to the germinal pathway, and the importance of each gross biochemical component to the reproductive process under different nutritional conditions all play a role in the adaptative strategies of a species to different habitats (Goodman, 1979).

Most information currently available with regard to reproductive strategies in bivalves proceeds from studies carried out in the natural medium (Beninger and Lucas, 1984; Napolitano et al., 1992; Urrutia et al., 1999; Li et al., 2000). Under such conditions, food availability varies during the period of sexual maturation, concomitant with other environmental conditions (e.g. temperature). It is therefore difficult to establish a direct relationship between a given state of energy balance (positive, negative, or zero) and the sexual maturation process or the utilisation of energy reserves. However, a few studies have

Table 9
Energy content (kJ ind^{-1}) of *R. philippinarum* after 48 experimental days

	Females (kJ ind^{-1})		Males (kJ ind^{-1})		Females (kJ ind^{-1})		Males (kJ ind^{-1})	
	14 °C L	18 °C L	14 °C L	18 °C L	18 °C H	22 °C H	18 °C H	22 °C H
Total energy	9.4	4.4	8.2	4.9	9.6	9.4	9.5	7.4
Protein	5.8±0.2	3.1±0.1	5.5±0.2	3.6±0.6	5.9±0.3	5.9±0.2	6.2±0.1	5.0±0.1
Carbohydrates	1.4±0.1	0.4±0.1	1.0±0.2	0.4±0.1	1.0±0.2	0.8±0.1	1.1±0.2	0.6±0.1
Lipids	2.2±0.2	0.9±0.1	1.7±0.2	0.9±0.1	2.7±0.3	2.7±0.3	2.2±0.2	1.8±0.2

Values are given as means±S.D.

isolated the influence of these environmental factors on the energetic balance of bivalves during the reproductive process (Borcherding, 1995; Navarro and Iglesias, 1995; Navarro et al., 2000; Rodríguez-Jaramillo et al., 2001) and on the administration of reserves (Martínez and Pérez, 2003).

Recently our group (Pérez-Camacho et al., 2003; Delgado et al., 2004), has studied, under controlled laboratory conditions, the influence exerted by different states of energy balance on the accumulation and utilisation of reserves throughout sexual maturation, including the evolution of different lipid classes of *R. decussatus*, and also examined differential responses regarding sex.

In our study, we observed that *R. philippinarum* conditioned at 18 °C (18L) shows higher protein content while at 14 °C (14L) the clams have a greater content of energy reserves (carbohydrates and glycogen), a similar pattern being shown in both sexes. A previous study (Delgado and Pérez-Camacho, 2007) using the same treatment (14L and 18L), with similar ingestion levels reported very slow gonadal development in male and female clams. The clams conditioned at low temperature (14L), had a positive energetic balance due to a lower metabolic expenditure. This was reflected in a considerable organic weight increase after 48 days. With high temperature (18L) the clams had negative energetic balance due to greater metabolic expenditure, although also displaying sexual development. An important loss of organic weight was observed after 48 days. In such a situation, the clams use their own reserves (carbohydrates and glycogen) for sexual development while in situations without food stress (positive energy balance) and low temperature (14 °C) an accumulation of reserves is produced (see Tables 1 and 4).

Strikingly dissimilar behaviour in biochemical composition was observed for the 18H and 22H treatments both with similar ingestion level, both with a positive energetic balance (but 2.5 times greater at 18 °C than at 22 °C), and a complete and similar gonadal occupation in each case (Delgado and Pérez-Camacho, 2007). Despite similar protein content, the highest levels of carbohydrate were observed at the lower temperature (18 °C). Glycogen was also higher for the 18 °C treatment, although the differences were significant ($p<0.05$) only in the males.

Laing and Child (1996) with *R. decussatus* and *R. philippinarum* and low temperature experiments (3, 6 and 9 °C) observed how the carbohydrates were converted into the main energy source, thereby decreasing notably in content. Their experiments, however, used a lower temperature range with possible dramatic decrease in ingestion rate and employed small juveniles, where the reproductive process did not interfere in the dynamics of the reserves.

Although the total lipids in *R. philippinarum* showed no significant differences in any treatment with the exception of males in group 14 °CL vs 18 °CL, they became apparent and related to sex when considering the individual lipid classes. Accordingly, with positive energetic balance (14L) the females showed greater contents of phospholipids, triglycerides, esters of sterols+waxes and sterols. No differences in free fatty acids could be determined. With the exception of triglycerides and esters of sterols+waxes, sexual differentiation disappeared with negative energetic balance (18L), as observed for *R. decussatus* (Delgado et al., 2004). For the clams subject to the 18H and 22H treatments (positive energetic balance) it is again the females which showed the greater content of energetic and structural lipids. The females conditioned at 18 °C had a higher phospholipids content whereas the males (18 °C) had a greater content of triglycerides.

When we compare the treatments with a greater temperature difference (14L vs 22H), both with different ingestion rates but similar energetic balances (about 10% en both cases), we observe that the females of both treatments increased their energy up to 9.4 kJ ind^{-1} after 48 days (the clams at the beginning of the experiment had an energy content of 6.8 kJ ind^{-1}) (see Tables 1 and 9). The protein contribution was similar (~62%). Beninger and Lucas (1984), however, noted a greater contribution of proteins to the maintenance energy requirements for this same specie in winter (74%). Differences were observed in the contribution of carbohydrates and lipids. For the 22H clams they supplied 8.5% and 28.7% to the total energy, respectively, and 14.8% and 23.4% for the 14L clams. This corresponded with a greater GOI (40% in females: Delgado and Pérez-Camacho, 2007) in the clams conditioned at 22 °C (22H) compared to about 20% for the clams cultured at 14 °C (14L).

For the males, similar differences as for the females were observed in the energy supply of carbohydrates for each treatment (12.2% for 14 °C (14L) and 8.1% for 22 °C (22H). Differences were also observed in the lipid contribution, although less pronounced than in the females (20.7 and 24.3% for the 14L and 22H treatments, respectively). Proteins contributed up to 67% of the total energy in both treatments (see Table 9).

There was no variation in lipid classes in the males between the 14L and 22H treatments despite the large disparity in the degree of sexual development. However in the females significant differences in lipid classes (phospholipids, triglycerides) were observed. This behaviour indicates the existence of differences related to sex. Such behaviour has already been observed for *R. decussatus* (Delgado et al., 2004) and other bivalves (Soudant et al., 1996; Li et al., 2000).

Our results show that abundant food permits the clams (*R. philippinarum*) their gonadal development and accumulation of reserves both in low and high temperature conditions. In low temperature situations gonadal development is slower and energy reserves are accumulated as carbohydrates. When the clams are at high temperatures, gonadal development is fast and complete, carbohydrates are consumed and lipids are accumulated.

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